Final Report for Period: 09/2007 - 08/2008 **Submitted on:** 01/29/2009

Principal Investigator: Taillefert, Martial . **Award ID:** 0433941

Organization: GA Tech Res Corp - GIT

Submitted By:

Taillefert, Martial - Principal Investigator

Title:

Molecular Mechanisms of Soluble Fe(III) Reduction by Metal-Reducing Members of the Genus Shewanella

Project Participants

Senior Personnel

Name: Taillefert, Martial

Worked for more than 160 Hours: Yes

Contribution to Project:

Name: DiChristina, Thomas

Worked for more than 160 Hours: Yes

Contribution to Project:

Post-doc

Graduate Student

Name: Meiggs, Deidre

Worked for more than 160 Hours: Yes

Contribution to Project:

Developed screening technique and other analytical methods to determine the chemical speciation during incubations

Name: Beckler, Jordon

Worked for more than 160 Hours: Yes

Contribution to Project:

Conducted incubations with wild type and mutant strains. Beckler became a graduate student this past year. He has investigated the production of soluble organic Fe(III) complexes by S. putrefaciens with various iron oxides as terminal electron acceptor. He is also optimizing a technique to separate organic-Fe(III) complexes for chemical characterization.

Name: Burns, Justin

Worked for more than 160 Hours: Yes

Contribution to Project:

Grew microorganisms, conducted incubations, and performed some of the mutagenesis work.

Name: Fennessey, Danielle

Worked for more than 160 Hours: Yes

Contribution to Project:

Performed the mutagenesis work for the siderophore mutants and most of the random mutagenesis of S. putrefaciens and S. oneidensis. She also conducted growth curves on different terminal electron acceptors and donors.

Name: Jones, Morris

Worked for more than 160 Hours: Yes

Contribution to Project:

Jones is responsible for the screening and incubations with the mutants. He prepares the voltammetric microelectrodes, validate their functionality, and calibrate them.

Undergraduate Student

Name: Aphivantrakul, Porntawee

Worked for more than 160 Hours: Yes

Contribution to Project:

Undergraduate biology student. He has learned molecular techniques and conducted basic growth curves for the project.

Name: Kieley, Ewelina

Worked for more than 160 Hours: Yes

Contribution to Project:

Biology student on the project for two semesters. Has learned molecular techniques and conducted basic growth curves for the project.

Name: Peart, Jason

Worked for more than 160 Hours: Yes

Contribution to Project:

Geochemistry student on the project for two semesters. Has learned voltammetric techniques and applied them to monitor production of soluble organic-Fe(III) in incubations with cells and different iron oxides

Name: Wilson, Patrick

Worked for more than 160 Hours: Yes

Contribution to Project:

Geochemistry student on the project for two semesters. Has learned voltammetric techniques and applied them to monitor production of soluble organic-Fe(III) in incubations with cells and different iron oxides

Name: Oliver, Brooke

Worked for more than 160 Hours: Yes

Contribution to Project:

Biology student on the project for two semesters. Has learned molecular techniques and conducted basic growth curves for the project.

Name: Miller, Morgan

Worked for more than 160 Hours: Yes

Contribution to Project:

Biology student on the project for two semesters. Has learned molecular techniques and conducted basic growth curves for the project.

Technician, Programmer

Other Participant

Research Experience for Undergraduates

Name: Miller, Lindsey

Worked for more than 160 Hours: Yes

Contribution to Project:

REU student during the summer, she has learned voltammetric techniques and basic microbiology techniques to conduct the screening of mutants.

Years of schooling completed: Junior

Home Institution: Other than Research Site

Home Institution if Other: University of Tennessee at Chattanooga

Home Institution Highest Degree Granted(in fields supported by NSF): Bachelor's Degree

Fiscal year(s) REU Participant supported: 2007

REU Funding: REU site award

Organizational Partners

Other Collaborators or Contacts

Activities and Findings

Research and Education Activities:

Dissimilatory Fe(III) reduction is central to a wide variety of environmentally and geochemically significant processes. Interestingly, the mechanisms of Fe(III) reduction are largely unknown. Much of the recent work on the molecular mechanism of microbial Fe(III) reduction has centered on the mechanism of solid Fe(III) reduction, yet has largely neglected the mechanism responsible for solubilization and subsequent reduction of soluble Fe(III) forms. Soluble Fe(III), under the form of organic-Fe(III) complexes, has been detected in significant concentrations in both freshwater and marine sediments and, in some cases, may represent a dominant oxidant in anaerobic environments. Simultaneously, incubations with iron-reducing bacteria of the genus Shewanella have demonstrated production of the same types of organic-Fe(III) complexes during the reduction of iron oxides. The main goal of this project was to determine the molecular mechanism by which metal-reducing members of the genus Shewanella solubilize and subsequently reduce iron oxides.

To accomplish this objective, several parallel investigations were conducted by both group. First, incubations with the wild type of S. oneidensis MR-1 and S. putrefaciens 200 strains in the presence of ferric citrate and different forms of iron oxides were used to study the kinetic of production of soluble organic-Fe(III) complexes. Second, growth of the wild type of both strains on alternative terminal electron acceptors in the presence of iron oxides was used to investigate whether formation of soluble organic-Fe(III) was putative to these organisms. Third, a voltammetric method and a size exclusion chromatography technique were optimized to quantify the production of soluble organic-Fe(III) by Shewanella and isolate these complexes for further chemical characterization. Fourth, mutants that lack the genes encoding known siderophores in S. oneidensis MR-1 and S. putrefaciens 200 were created using directed mutagenesis to determine whether organic-Fe(III) complexes were siderophore complexes. Fifth, a screening technique was developed and applied to test the ability of randomly mutagenized cells to produce soluble organic-Fe(III). More than 4000 random mutations were performed with S. oneidensis MR-1 to screen for a Fe(III) solubilization deficient mutant. Using this screening technique, four mutants of the wild type S. oneidensis MR-1 were found and investigated in batch reactors in the presence of different forms of ferric iron and various electron donors. These soluble Fe(III)-deficient mutants, along with other known mutants of S. oneidensis MR-1, were further tested for their ability to respire on alternative terminal electron acceptors in the presence of fumarate, lactate, or pyruvate as electron donor. Finally, a clone bank was generated and genes deleted from S. oneidensis MR-1 were reinserted into the four mutants to identify the genes deleted in these mutants.

Findings: (See PDF version submitted by PI at the end of the report)

Training and Development:

Beckler, Peart, and Wilson (EAS undergraduate students) as well as L. Miller (REU student) were trained in geomicrobiology. They learned how to use appropriate clean techniques to work with microorganisms and are now proficient in the use of voltammetric techniques. Aphivantrakul, Kieley, B. Miller, and Oliver (Biology undergraduate students) learned how to use appropriate mutant generation techniques and conventional techniques used in biology to characterize microorganisms (plate bacterial counts, growth curves, rate measurements, etc.).

The graduate students were trained in the use of genetic (Burns, Fennessey) and voltammetric (Beckler, Jones, Meiggs) techniques. Beckler and Meiggs also learned how to separate organic-Fe(III) complexes using a size exclusion chromatography technique. The graduate students are also responsible for the generation of three manuscripts that have been recently published or submitted. Their daily interaction has clearly benefited their work.

Outreach Activities:

Taillefert and DiChristina's groups gave tours of their laboratories to high school and middle school students about three times of year during the four years of this project. During these tours, students are exposed to the field of geomicrobiology and the role of microorganisms in the environment, in the past and the present.

Journal Publications

Taillefert, M., J. Beckler, E. Cary, J. Burns and T. DiChristina, "Shewanella putrefaciens produces an Fe(III)-solubilizing ligand during anaerobic respiration on insoluble Fe(III) oxides", Journal of Inorganic Biochemistry, p. 1760, vol. 101, (2007). Published, 10.1016/j.jinorgbio.2007.07.020

DiChristina, T., D. Bates, J. Burns, M. Adiga and C. Haller., "AQDS electron shuttling pathway rescues the Fe(III) and Mn(IV) respiratory deficiencies of type II protein secretion (gspD) mutants of Shewanella oneidensis MR-1", Journal of Bacteriology, p. , vol. , (2007). Submitted,

DiChristina, T., J. Fredrickson and J. Zachara, "Enzymology of electron transport: Energy generation with geochemical consequences", Reviews in Mineralogy and Geochemistry, p. 27, vol. 59, (2005). Published,

M. Jones, C. Fennessey, T. J. DiChristina, M. Taillefert, "Design and application of a rapid screening technique for isolation of iron(III) solubilization-deficient mutants of Shewanella oneidensis MR-1", Environmental Microbiology, p. , vol. , (2008). Submitted,

C. Fennessey, M. Jones, M. Taillefert, T. J. DiChristina, "In-frame deletion mutants of S. oneidensis MR-1 siderophore biosynthesis genes produce an Fe(III)-solubilizing organic ligand under anaerobic (but not aerobic) conditions", Journal of Bacteriology, p. , vol. , (2008). Submitted,

Books or Other One-time Publications

DiChristina, T., D. Bates, J. Burns, J. Dale and A. Payne, "Microbial metal reduction by members of the genus Shewanella: novel strategies for anaerobic respiration.", (2006). Book, Published
Editor(s): L. Neretin
Collection: Biogeochemistry of Anoxic Marine Basins
Bibliography: Kluwer Publishing Co., Dordrecht, NL.

Web/Internet Site

Other Specific Products

Contributions

Contributions within Discipline:

The combination of mutagenesis techniques and voltammetric techniques provides a unique opportunity to investigate the mechanisms of iron reduction by Shewanella. Our studies demonstrate that organic-Fe(III) complexes detected during the reduction of iron oxides are not generated by siderophores. In addition, the fact that the rates of production of organic-Fe(III) complexes varies linearly with the rates of iron reduction in four new mutants that are good candidates for a deficiency unique to metal reduction as well as two well-characterized mutants of Shewanella is additional evidence that the solubilization pathway plays an important role in anaerobic respiration of iron oxides. Identification of the genes deleted in these mutants will provide new insights on their role in the Fe(III) solubilization pathway.

Contributions to Other Disciplines:

Understanding the molecular processes involved in anaerobic respiration of iron oxides may benefit other disciplines, including environmental engineering and oceanography. Dissimilatory iron reduction is central to a wide variety of environmentally significant processes, including the reductive immobilization of toxic metals (e.g., U, Tc, Cr) and the reductive decomposition of xenobiotic compounds by Fe(II). Understanding the molecular processes by which Fe(II) is generated may have an important impact on remediation strategies. Furthermore, dissimilatory iron reduction plays an important, yet unappreciated, role in the cycling of carbon in marine sediments. The lack of functional molecular probes and the difficulty in cultivating iron-reducing bacteria has unfortunately undermined the role of dissimilatory iron reduction in these environments. Gaining insights into the molecular mechanism of iron reduction may help in the design of new functional molecular probes or identify new tracers of this process. In this respect, the finding that formation of voltammetrically labile intermediate organic-Fe(III) complexes is required in dissimilatory iron reduction provides a new strategy to trace iron-reducing bacteria in marine sediments with voltammetric techniques.

Contributions to Human Resource Development:

The REU supplement received for this project has benefited the education of eight undergraduate students across geochemistry and microbiology disciplines during this project. This cross-disciplinary experience has proven extremely valuable to these students. They not only have learned how to communicate and collaborate, they also benefited from each other's expertise to tackle a complex scientific problem. About 50% of these students have used this experience as a motivation factor to continue their studies at the graduate level.

Contributions to Resources for Research and Education:

During Year 1, Taillefert has presented his group's findings at the National Meeting of the American Chemical Society (ACS) in Washington, DC and was an invited speaker at the Goldschmidt conference in Moscow, ID. DiChristina has presented his group's findings in poster format at the American Society for Microbiology (ASM) National Meeting in Atlanta and at the International Union of Microbiological Societies (IUMS) International meeting in San Francisco. DiChristina was an invited speaker at the Goldschmidt conference in Moscow, ID, at the Clay Mineralogical Society (CMS) Annual meeting in Richland, WA and at the Soil Science Society of America (SSSA) National meeting in Seattle.

During Year 2, Taillefert was an invited speaker at the Gordon Research Conference in Environmental Sciences: Water at Holderness School in New Hampshire. Taillefert has also organized a Geomicrobiology session at the ACS Meeting in Atlanta, GA. This session included 20 abstracts and regrouped both geochemists and biologists interested in geomicrobial processes. DiChristina's graduate student Christine Fennessey presented her findings in poster format at the ASM National Meeting in Orlando, FL. DiChristina presented his group's findings as an invited speaker at the ASM National Meeting in Orlando, FL, at the Environmental Bioinorganic Chemistry Gordon Conference in New London, NH, at the Mineralogical Society of America (MSA) National Meeting in Berkeley, CA and at the ACS National Meeting in San Francisco, CA.

During Year 3, Taillefert's graduate student, Jordon Beckler presented his findings at the Geological Society of America Southeastern Section Meeting in Savannah, GA. Taillefert was a plenary speaker at the 11th Workshop on Progress in Analytical Methodologies for Trace Metal Speciation in M³nster, Germany. DiChristina's graduate student Christine Fennessey presented her findings in poster format at the ASM National Meeting in Toronto, Canada. DiChristina presented his group's findings as an invited speaker at Cornell University, the University of Minnesota, and the Gordon Research Conference on Applied and Environmental Microbiology in South Hadley, MA.

During Year 4, Taillefert was invited to present his group's findings at the ACS National Meeting in Philadelphia, PA, at the University of South Carolina, and at the Telluride Workshop on the biogeochemical processes of the iron cycle: From microbes to mineral surfaces in Telluride, CO. DiChristina was invited to present his group's findings at Harvard University, at the ACS National Meeting in Philadelphia, PA, at the Gordon Research Conference on Environmental Bioinorganic Chemistry in Waterville Valley, NH, and at the Telluride Workshop on the biogeochemical processes of the iron cycle: From microbes to mineral surfaces in Telluride, CO. Finally, DiChristina's graduate student, Justin Burns, presented his findings at the ASM National Meeting in Boston, MA.

Contributions Beyond Science and Engineering:

Categories for which nothing is reported:

Organizational Partners

Any Web/Internet Site

Any Product

Contributions: To Any Beyond Science and Engineering

2.1 Findings

Growth of *S. putrefaciens* on different forms of Fe(III) as terminal electron acceptor. Growth incubations of *S. putrefaciens* with different forms of iron oxides in batch reactors confirmed that soluble organic-Fe(III) is produced with all iron oxides (Taillefert et al., 2007). It is well known that the reactivity of iron oxides during microbial reduction depends on the crystallinity of the iron oxides, possibly linked to the surface area available for contact with the microorganism. Initial rates of production of soluble Fe(III) correlated well with rates of iron reduction, suggesting that these complexes are intermediates in the reduction of iron oxides. In turn, rates of soluble Fe(III) and iron reduction were essentially invariable when normalizing the initial rates of iron reduction and iron solubilization to the surface area of each substrate. These results confirm the intrinsic chemical reactivity of the iron substrate is not responsible for the different rates of iron reduction observed with minerals of different crystallinity and suggest that the organic ligand synthesized by *Shewanella* is strong enough to solubilize even the less reactive iron substrates (i.e. hematite and magnetite). These results also suggest that the solubilization step is required for iron reduction, likely because this step destabilizes the iron oxides in order to lower the activation energy required to facilitate the reduction of Fe(III). Additional experiments with a mutant of *S. oneidensis* MR-1 that lacks GspD, a central component of the Type II secretion system which transfers the iron reductase to the outer membrane surface, revealed that ΔgspD is not capable of producing soluble organic-Fe(III) complexes, but the strain complemented with the portion of gene previously deleted restored production of soluble organic-Fe(III) as well as Fe(III)-reduction activity (unpublished results). Other experiments with a mutant of *S. oneidensis* MR-1 that lacks MtrC, a cytochrome positioned on the outer membrane and hypothesized to be part of the terminal reductase complex in *Shewanella* species, demonstrate partially impaired iron reduction activity but no production of soluble organic-Fe(III) in ΔmtrC (unpublished results). These results suggest that the organic ligand may be secreted by the Type II secretion system and that MtrC is involved in the generation of the soluble Fe(III) complexes as well as in iron reduction.

Growth of *S. oneidensis* on alternate terminal electron acceptors in the presence of iron oxides. Growth of *S. oneidensis* on dissolved oxygen, nitrate, or TMAO in the presence of amorphous iron oxides revealed that formation of soluble organic Fe(III) complexes is not putative during growth of *Shewanella* species. Voltammetric signals for soluble organic-Fe(III) complexes were not detected in any incubation conducted in the presence a competitive terminal electron acceptor to iron respiration. These results confirm that the production of the organic ligand is unique to the anaerobic respiration on iron oxides (Jones et al., 2009).

Quantification and isolation of soluble organic-Fe(III) complexes. Voltammetric signals for soluble organic-Fe(III) complexes depend on the composition of the organic ligand because the entire complex is reduced at the electrode surface. Thus, voltammetric measurements cannot be used to quantify organic-Fe(III) complexes directly because the nature of the organic ligand produced by the organism is unknown, and other techniques are needed to calculate rates of iron solubilization. Unfortunately, quantification of soluble organic-Fe(III) using a conventional speciation scheme with colorimetric techniques has also proven difficult. The main problems reside in that solid Fe(III) concentrations used are high, the reactive fraction of solid Fe(III) unknown, and filtration techniques may cause artifacts in the separation of the different phases. During this project, competitive ligand exchanged techniques have being optimized to quantify

soluble organic-Fe(III) complexes directly by voltammetry. This technique is based on the addition of a known organic ligand that competes with the natural ligand for iron complexation. The choice of the competitive ligand is difficult, as its complexation capacity should be similar, but higher, than that of the natural ligand. We have tested different organic ligands to optimize the competitive ligand concentration in the medium used during the incubations. Simultaneously, we have optimized a size exclusion chromatography technique to isolate the organic-Fe(III) complexes from the medium for further characterization. These studies are still ongoing, but preliminary results show that soluble organic-Fe(III) complexes are produced in hundreds of micromole per liter in typical incubations with 10^8 cells ml^{-1} (unpublished results).

Siderophore mutants in *S. oneidensis* and *S. putrefaciens*. The objective of this part of the project was to demonstrate that the soluble-organic Fe(III) complexes detected in incubations with *Shewanella* species were not siderophore complexes. Most organisms secrete Fe(III)-solubilizing siderophores for Fe(III) assimilation, as iron is a required nutrient in nearly every living organism. To confirm that the complexes observed by voltammetry were not produced by siderophores for iron acquisition, deletion mutants of SO3031 – a siderophore synthesis gene in *S. oneidensis* MR-1 and *S. putrefaciens* 200 – were prepared with both organisms (Fennessey et al., 2009). The deletion was confirmed using PCR amplification of the region. These mutants were then tested on chrome azurol-S plates (CAS), a standard colorimetric assay for the detection of siderophore production, with the Arnow test, an assay to detect catecholate type siderophores, and the ferric perchlorate test, an assay to detect hydroxamate and aerobactin-type siderophores, to confirm the deletion of all siderophore biosynthesis. Under aerobic conditions, the ΔSO3031

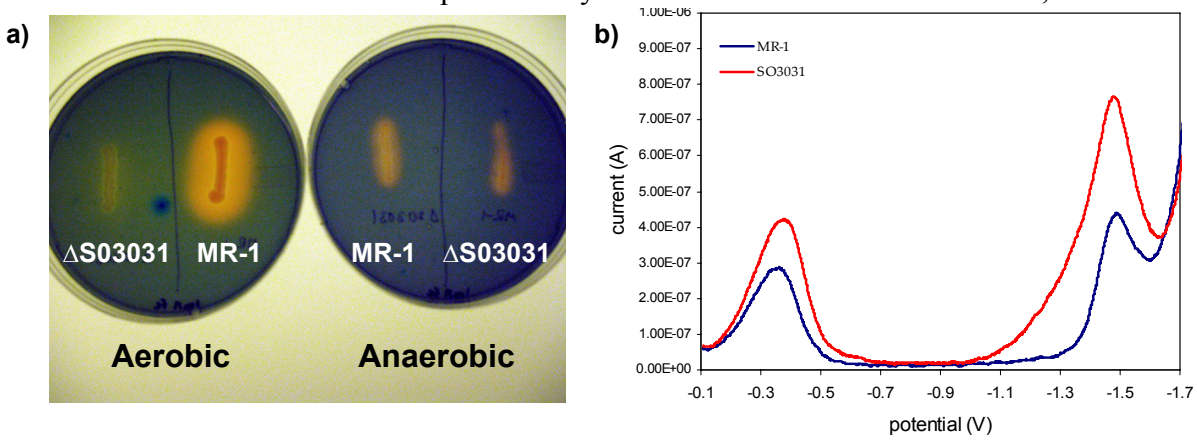


Figure 1. Chrome azurol S (CAS) assay of *S. oneidensis* MR-1 and the targeted siderophore mutant ΔSO3031 grown on agar plates in both aerobic and anaerobic conditions. a) Yellow halo indicate removal of iron from the plate by siderophore. Under aerobic conditions the yellow halo surrounding ΔSO3031 is much smaller than the halo of MR-1. Under anaerobic conditions the halos are the same for both ΔSO3031 and MR-1 and moderate in size. b) Voltammetric scans of MR-1 and ΔSO3031 incubated anaerobically in batch reactors with 32 mM amorphous iron oxides. Voltammetric response of the siderophore mutant and wild-type are similar, which indicates that siderophores do not contribute significantly to the production of organic-Fe(III).

mutant of *S. oneidensis* MR-1 yielded negative results on CAS plates compared to wild-type cells, confirming successful cessation of siderophore production (Figure 1a for *S. oneidensis*). Under anaerobic conditions, however, both mutants and wild-types displayed a positive CAS phenotype. These results indicate that under anaerobic conditions, the cells produce a Fe(III)-chelating compound that is distinct from a siderophore. Voltammetric scans also revealed that the ΔSO3031

mutants behaved almost identically to the wild-type organism (Figure 1b), confirming that the soluble Fe(III) and Fe(II) peaks observed are not a result of Fe solubilization by a siderophore. Finally, analysis of the growth capabilities of the SO3031 deletion mutant in *S. oneidensis* MR-1 with both amorphous iron oxides and soluble ferric citrate with a variety of electron donors in a range of medias revealed that the ΔSO3031 mutant of *S. oneidensis* MR-1 is capable of growth at wild-type rates, demonstrating that the siderophore is not involved in anaerobic Fe(III) respiration. These data appear to indicate that the molecules synthesized by *S. oneidensis* MR-1 for solubilization of Fe(III) during respiration are distinct from those produced for Fe(III) assimilation (Fennessey et al., 2009).

The deletion of the SO3031 analog in *S. putrefaciens* 200, however, resulted in growth deficiencies on multiple electron acceptors, including both solid and soluble Fe(III) (unpublished results). This mutant did also not produce soluble organic-Fe(III) voltammetric signals when incubated with hydrous ferric oxides. However, it is presumed that this incapacity to solubilize solid Fe(III) is due to the mutant's inability to survive without siderophore when incubated with solid Fe(III) as sole electron acceptor. The differences between the SO3031 deletion in *S. oneidensis* MR-1 and its analogous deletion in *S. putrefaciens* 200 represent an interesting example of the genetic dissimilarities between two closely related strains which bears further investigation.

The open reading frame containing the siderophore biosynthesis genes also contains a gene annotated as a ferric reductase (SO3034). Because it is on the same ORF as the siderophore biosynthesis genes, it was presumed to be a ferric reductase specific to siderophore-Fe(III) species. To determine whether this reductase gene is specific to siderophores of if it plays a major role in reduction of soluble Fe(III), SO3034 was deleted in both *S. oneidensis* MR-1 and *S. putrefaciens* 200. Anaerobic incubation of these mutants with a variety of electron acceptors resulted in growth and respiration at wild-type rates. Additionally, incubations of the mutants with hydrous ferric oxides followed by square-wave voltammetry screening resulted in no significant difference from wild-type production of either soluble Fe(III) or Fe(II) (unpublished results). These findings indicate that the ferric reductase involved in the reduction of siderophore-bound Fe(III) is distinct from the ferric reductase involved in Fe(III) respiration.

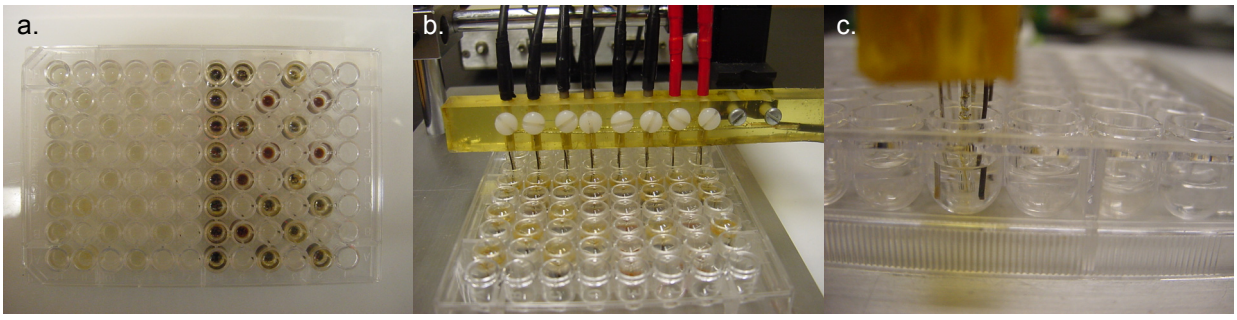
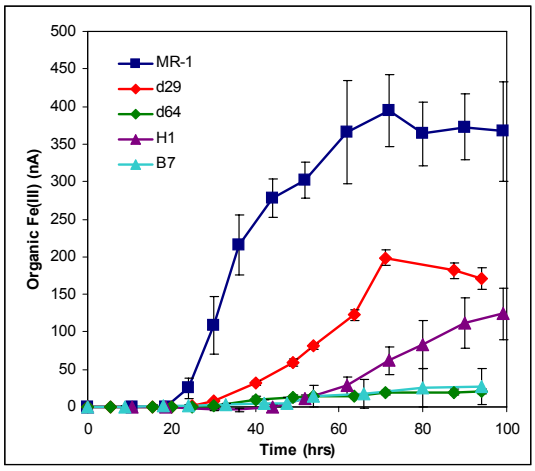


Figure 2. Voltammetric assay for mutant screening. a. 96-well microtiter plates filled with ferric citrate solution (columns 2 to 6) and hydrous ferric oxides (columns 7 to 12). b. View of the eight individually-addressed Hg/Au voltammetric microelectrodes with interconnected Ag/AgCl reference and Pt counter electrodes. c. Close up on one of the wells with the three electrodes.

Voltammetric screening of soluble organic-Fe(III)-deficient mutants. A screening technique (Figure 2) was developed to monitor soluble organic-Fe(III) production during the

anaerobic respiration on iron oxides in liquid cultures with cells mutagenized randomly with ethyl methanesulfonate (EMS). To screen large colonies of mutated cells, standard 96-well microtiter plates were used to grow the mutants (Figure 2a), and a array of eight mercury gold amalgam (Au/Hg) voltammetric microelectrodes and eight permanent reference and counter electrodes was built to screen the cells row by row (Figure 2b and c). The electrode array of counter electrodes were made by soldering eight pieces of 0.5 mm platinum wire together every 0.8 cm along a piece of striped stranded coaxial cable such that the distance between each wire was equal to the distance between the wells of a 300 µL 96-well tray. A similar array of reference electrodes was fabricated with 0.5 mm silver wire. The counter and reference arrays were embedded in epoxy such that 2 cm of the wires extended outside of the mold (Figure 2b). Eight holes were drilled between the counter and reference electrodes to position the Au/Hg working electrodes in the middle of each well (Figure 2b). To screen the colonies, the electrode array was positioned in each individual well using a three-dimensionally adjustable micromanipulator (Figure 2c), and voltammograms were collected successively at each working electrode using an automatic software. Using that system, it was possible to measure both soluble organic-Fe(III) and Fe(II) simultaneously and complete measurements in each well in less than a minute.



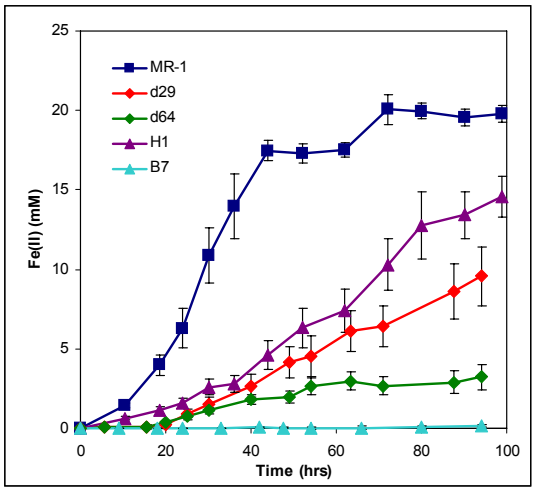


Figure 3. a) Voltammetric organic-Fe(III) and b) Total Fe(II) produced as a function of time in batch reactors containing 32 mM amorphous iron oxides in Westlake media with lactate as electron donor in the presence of 2 10^7 cells/ ml. MR-1 is the wild-type strain. d29, d64, H1, and B7 are the four random mutants discovered by the screening technique.

The voltammetric assay optimized during year 1 and 2 was used to screen approximately 4000 mutants during year 3 and 4. Each screening assay consisted of incubating 32 mM hydrous ferric oxides and 10^8 mutant cells in separate wells of all the columns from row 1 to 10. Chemical controls were also inserted in all the columns of row 11, while the wild type *S. oneidensis* MR-1 was used as a positive control in all the columns of row 12. The cells were incubated for 72 hours before screening by square wave voltammetry. The surface area of the voltammetric peak for soluble organic-Fe(III) detected by each electrode normalized to the surface area of the voltammetric signal of a 200 µM Mn(II) standard at the same electrode were used to compare data between each electrode. Results were reported as positive if the normalized surface area of a particular well was greater than 75% of the average surface area reported at the same electrode in the presence of *S. oneidensis* MR-1. Negative results represented signals that were smaller than 25% of the average surface area reported at the same electrode in the presence of MR-1. Out of the 4000 mutants investigated, four were found to be impaired in their ability to produce soluble organic-

Fe(III) and reduce iron oxides (Jones et al., 2009).

These mutants were further investigated in batch reactor incubations in the presence of hydrous ferric oxides and ferric citrate and compared to parallel incubations conducted with *S. oneidensis* MR1 (Figure 3). In typical anaerobic batch incubations, the production of Fe(II) by *S. oneidensis* MR-1 is immediate and accompanied by the formation of organic-Fe(III) in solution after a small phase lag. The four random mutants isolated during this study behave much differently. Mutant d29 grows after a longer lag time and produces organic–Fe(III) and Fe(II) with lower rates and lower intensity (55-60% at steady-state) than *S. oneidensis* MR-1. Mutant d64 and B7 are both severely impaired in their Fe(III) reduction activity and ability to produce soluble organic Fe(III). At steady-state, mutant d64 displays approximately 17% of the iron reduction activity and 7% of soluble organic-Fe(III) production of *S. oneidensis* MR-1, while mutant B7 only reaches 7% of the iron reduction activity and 1% of the organic-Fe(III) production of *S. oneidensis* MR-1. In turn, mutant H1 displays a long lag time before production of organic–Fe(III), despite the fact that Fe(II) is detected almost immediately upon inoculation, and, at steady-state, 33% of soluble organic-Fe(III) and 76% of iron reduction activities are typically recorded with this mutant compared to *S. oneidensis* MR-1.

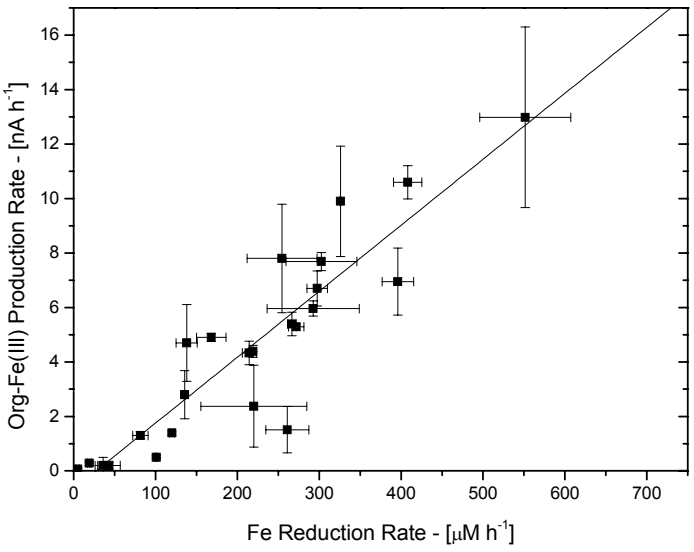


Figure 4. Initial rates of organic-Fe(III) production against initial rates of Fe(II) production in triplicate incubations with *S. oneidensis* MR-1 and *S. oneidensis* random mutants d29, d64, H1, and B7. The linear relationship between organic-Fe(III) and Fe(II) production during dissimilatory iron(III) reduction suggests that soluble organic-Fe(III) is an intermediate in the reduction of iron oxides.

Initial rates of organic-Fe(III) production measured in incubations with *S. oneidensis* MR-1, random mutants d29, d69, B7, and H1, as well as with the ΔgspD and ΔmtrC mutants of *S. oneidensis* MR-1 correlate linearly (R^2 = 0.9) with initial rates of iron reduction (Figure 4). The linear relationship between the rate of production of soluble organic-Fe(III) and the rate of production of Fe(II) indicates that the presence of the organic-Fe(III) complexes is necessary for iron reduction. These findings suggest that bacterial respiration of solid Fe(III) oxides proceeds through a non-reductive Fe(III) solubilization step prior to the reduction of the produced soluble organic Fe(III) complex. Unfortunately, it is difficult to apply significance to the slope of this relationship (24(+/-1) pA organic Fe(III) per μM Fe(II)) as the Fe(III)-ligand complexes cannot be directly quantified by voltammetry. Voltammetric measurements using the competitive ligand exchange method are currently performed to quantify the relationship between iron solubilization and iron reduction.

Altogether, these experiments are the first to demonstrate that anaerobic respiration on solid iron oxides involves an intermediate solubilization step that appears to be required. In addition, this study demonstrated that siderophores produced for iron assimilation were not involved in the

production of the intermediate soluble organic-Fe(III) complexes during anaerobic respiration on iron oxides. Finally, this study identified for the first time mutations unique to the chelation pathway involved in the solubilization of iron oxides. The non-reductive solubilization of solid Fe(III) oxides to soluble organic Fe(III) intermediates presents advantages over the direct contact pathways proposed previously. Fe(III) solubilization may increase rates of reduction, the reduction potential of Fe(III), and facilitate access to Fe(III). If a group of cells works in concert and cycles the organic ligands, the advantages of solubilization may offset the biosynthetic cast of ligand production. Finally, results from these incubations indicate that organic Fe(III) signals reach steady state before Fe(II), suggesting that the microbially-produced ligands maybe recycled after reduction to generate fresh organic-Fe(III) and limit energy costs associated with the biosynthesis of the ligand.

2.2 Training and Development

Beckler, Peart, and Wilson (EAS undergraduate students) as well as Miller (REU student) were trained in geomicrobiology. They learned how to use appropriate clean techniques to work with microorganisms and are now proficient in the use of voltammetric techniques. Aphivantrakul and Kieley (Biology undergraduate students) learned how to use appropriate mutant generation techniques and conventional techniques used in biology to characterize microorganisms (plate bacterial counts, growth curves, rate measurements, etc.).

The graduate students were trained in the use of genetic (Burns, Fennessey) and voltammetric (Beckler, Jones, Meiggs) techniques. Beckler and Meiggs also learned how to separate organic-Fe(III) complexes using a size exclusion chromatography technique. The graduate students are also responsible for the generation of three manuscripts that have been recently published or submitted. Their daily interaction has clearly benefited their work.

2.3 Outreach Activities

Taillefert and DiChristina's groups gave tours of their laboratories to high school and middle school students about three times of year during the four years of this project. During these tours, students are exposed to the field of geomicrobiology and the role of microorganisms in the environment, in the past and the present.